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Inflammasome expression and cytomegalovirus viremia in critically ill patients with sepsis

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ABSTRACT

Background: CMV viremia is a contributor to poor outcomes in critically ill patients with sepsis.
Objectives: To assess the expression levels of genes encoding inflammasome-related proteins in the development of CMV viremia in critically ill patients with sepsis.
Study design: A cohort of CMV-seropositive critically ill patients with sepsis due to bloodstream infection underwent weekly testing for CMV viremia. Blood samples to evaluate *mRNA* levels of genes encoding *CASP1, ASC, NLRP1, NLRP3,* and *NLRP12* were collected at the time of enrollment. Clinical outcomes were assessed at 30 days or until death/discharge from ICU.
Results: CMV viremia was documented in 27.5% (8/29) of the patients, a median of 7 days after the onset of bacteremia. Patients with sepsis who developed CMV viremia had higher *CASP1* although this was not statistically significant (relative mean 3.6 vs 1.8, p = 0.13). Development of high grade CMV viremia however, was significantly associated with *CASP1*; septic patients who developed high grade CMV viremia had significantly higher *CASP1*than all other patients (relative mean 5.5 vs 1.8, p = 0.016).
Conclusions: These data document possible involvement of inflammasome in the pathogenesis of CMV. Regulating the host immune response by agents that target these genes may have implications for improving CMV-related outcomes in these patients.

1. Background

Cytomegalovirus (CMV) is a member of the herpesvirus family and a significant human pathogen. CMV seropositivity indicative of latent infection is common in general population with rates of ~60–70% in the United States and higher in other parts of the world [1]. The virus establishes latency with lifelong persistence in infected individuals without overt or adverse sequelae. Latent infection, however, has the potential to reactivate and produce lytic virus. Although frequently documented in immunosuppressed patients, CMV viremia is exceedingly rare in immunologically competent individuals [2]. However, it has been recognized that critically ill patients who are otherwise immunocompetent are also at risk for CMV and that reactivation of CMV in these patients is a contributor to poor outcomes [3–5]. CMV viremia occurs in 15–30% of these patients, and sepsis-associated changes in the host innate immunity and upregulation of inflammatory cytokines are proposed to be the basis for the development of viremia [4,5].

NOD-like receptors (NLR) are intracellular sensors to detect microbes and danger molecules, and enhance innate immune responses [6,7]. Some NLR proteins are involved in the assembly of a cytosolic multiprotein complex termed the “inflammasome.” Components of inflammasomes typically include a NLR (e.g., NLRP1 or NLRP3, serving as a sensor), pro-caspase-1 (CASP1), and apoptosis-associated speck-like protein (ASC), an adapter protein bridging between NLR and pro-caspase-1 [8–12]. Once the inflammasome complex is formed, pro-caspase-1 goes through self-cleavage to become active. A key function of caspase-1 is to cleave pro-IL-1β and pro-IL-18 to mature inflammatory cytokines IL-1β and IL-18, which are released to extracellular spaces by pyroptosis, an inflammatory cell death [9,13]. IL-1β is one of the most potent proinflammatory cytokines for the generation of systemic and localized responses to sepsis [9,14,15]. Thus, inflamma-

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somes form molecular scaffolding and function as critical inducers of inflammatory responses [8,12].

Although inflammation is an essential component of protective responses to pathogens, proinflammatory pathology may have detrimental effects including reactivation of CMV [16,17]. Detection of IL-1β through IL-1 receptor, which activates NF-κB, leads to CMV reactivation [18,19]. However, the contribution of inflammasomes in the pathogenesis of CMV is not fully understood. The goals of the study herein were to assess expression levels of genes encoding inflammasome-related proteins, i) in the overall population of critically ill patients with sepsis, ii) as predictors of outcomes in septic patients, iii) and development of CMV viremia in a prospectively followed cohort of critically ill patients with sepsis.

2. Study design

2.1. Study population

We performed a prospective and observational study involving critically ill patients with sepsis due to bloodstream infections. The clinical characteristics and patient outcomes associated with CMV viremia in these patients have recently been reported [22]. Bloodstream infections in the patients were conducted between December 2008 and June 2014 at the intensive care units of two University-affiliated medical centers [22]. This study was approved by the institutional review board, and informed consent in a written form was obtained from the patients or their legally authorized representatives. Inclusion criteria included i) age ≥18 years and ii) hospitalization in the ICU at the time of or within 48 h of the onset of bloodstream infection. Patients with sepsis met previously defined International Sepsis Definition criteria [23] and had microorganism-positive blood culture with at least a single microbial species other than coagulase-negative staphylococci, the *Bacillus* species and diphtheroids based on the criteria for bacteremia by Centers for Disease Control and Prevention [24]. Bacteremia due to intravascular catheters or bacteremia without a documented source was regarded as primary bacteremia [24]. We considered patients to be immunocompetent if they had no known or overt evidence of immunosuppression. Patients were excluded if they were immunosuppressed as a result of; organ transplantation or hematopoietic stem cell transplantation, human immunodeficiency virus infection, receipt of antiviral medications with activity against CMV (i.e. ganciclovir or valganciclovir), and receipt of iatrogenic immunosuppressive agents, such as prednisone at a dose of > 0.5 mg/kg/day for at least 2 weeks prior to onset of sepsis, tumor necrosis factor antagonists, methotrexate, cancer chemotherapy within 4 weeks prior to the onset of sepsis.

2.2. Clinical assessments

Characteristics of the study population included demographic data, medical history, clinical and laboratory data were abstracted systematically in a data collection form. Organ failure was assessed upon enrollment into the study (considered as baseline) using Sequential Organ Failure Assessment (SOFA) score on a scale ranging from 0 to 4 for each of the six major organ systems, for an aggregate score of 0–24, with higher scores indicating more severe organ dysfunction [25]. Multiple organ failures were defined as ≥2 organ failure. Patients were followed for 30 days from the date of positive blood culture or until death or discharge from the ICU.

2.3. Laboratory assessments

2.3.1. CMV assays

After obtaining informed consent, a screening test for CMV antibody to determine CMV serostatus was performed using a commercially available immunoassay per manufacturer's instructions (Bio Merieux Vidas CMV IgG). Patients seropositive for CMV were enrolled and underwent weekly assessment for CMV viremia for 30 days from the date of positive blood culture or until death or discharge from the ICU. CMV assays were performed using a non-commercial whole blood quantitative real-time PCR as previously reported [26,27]. Briefly, DNA was extracted from 200 µl of whole blood into 150 µl elution volume, of which 5 µl was used per PCR reaction The primer sequences tested were: US17 forward 5′-CGATCAAGAACGCGATAACG-3′; US17 reverse 5ᶦACCGTCGATGGCAGGTCAT-3′; US17 probe 6FAM-CGA TCA CAA ACA GCG-MGB; UL54 forward 5′-CGCAGTCTACCTCGATATCACAA-3; UL54 reverse 5′-TGCTCCGTGAATCGTTACGA-3′; UL54 probe 6FAM-CCCTGCTGCCGCCA-MGB. Each real-time PCR was performed in 25 µl reaction volume and consisted of 1 × Taqman PCR master mix (Applied Biosystems, CA), 0.5 µM of forward and reverse primers, 0.2 µM of probe and 5 µl of extracted DNA. Viral load represented CMV DNA copies/mL. The assay's range for the detection of CMV was 50–10^6 copies/mL. High-grade viremia was defined as CMV DNA PCR copies > 500 copies/mL [27]. The results of CMV PCR assays were not used for clinical care.

2.4. Sample collection and processing

Blood samples from patients with sepsis were collected for the assessment of *CASP1, ASC, NLRP1, NLRP3,* and *NLRP12* mRNA levels were collected immediately upon enrollment into the study. Approximately 10 ml of whole blood samples were collected in EDTA-containing tubes and filtered immediately through LeukoLOCK Total RNA Isolation System (Thermo Fisher Scientific, Waltham, MA) that captures the total leukocyte population while eliminating plasma, platelets and red blood cells. The filter was flushed with a phosphate-buffered saline solution to remove residual red cells and then with RNA*later*$^{(R)}$ to stabilize leukocyte RNA. The filter with stabilized leukocytes was sealed and stored at −80 °C per manufacturer's instructions.

2.5. Evaluating mRNA levels by qPCR

Quantitative PCR was performed as previous performed [28,29]. cDNA was prepared from mRNA by reverse transcription (QuantiTect Reverse Transcription Kit, Qiagen). mRNA expression levels were determined by real-time PCR using the *ΔΔCt* method with SYBR™ Green master mixes (Applied Biosystems) with *ACTB* an internal control. Samples were run triplicate. Primer sequences used in analyses were *CASP1* (forward, 5′- GGATATGGAAACAAAAGTCGGC-3′; reverse 5′-CATTGTCATGCCTGTGATGTC-3′), *ASC* (forward, 5′- TCACCGCTAACGTGCTG-3′; reverse 5′- TGGTCTATAAAGTGCAGGCC-3′), *NLRP1* (forward, 5′- CCGCTGACCCCACTTTATATG-3′; reverse 5′- CAACGTAGAACTCCGAGAACAG -3′), *NLRP3* (forward, 5′- GTGTTTCGAATCCCACTGTG-3′; reverse 5′-TCTGCTTCTCACGTACTTTCT-3′), *NLRP12* (forward, 5′- TGACAGGAAATGCACTGGAG-3′; reverse 5′-GGTTCACACTGAGAGTTGAGG-3′), and *ACTB* (forward, 5′- ACCTTCTACAATGAGCTGCG-3′; reverse 5′CCTGGATAGCAACGTACATGG3′). PCR reaction was initiated with a step at 95C for 10 min and performed with 40 cycles of denaturation at 95C for 15 s and annealing/extension at 60C for 1 min. A majority of the qPCR results showed the range in the Ct value between 17 and 23. The highest Ct values detected the study was 26.54. By confirming the kinetics of relative fluorescent levels vs. PCR cycles, we concluded that all the samples used in this study had mRNA levels above the detection limit. We also evaluated the RQ-Min and RQ-Max values from technical triplicate [RQ-Min = $2^{-(\Delta\Delta Ct\ +\ T^{*}SD(\Delta Ct))}$, RQ-Max = $2^{-(\Delta\Delta Ct\ -\ T^{*}SD(\Delta Ct))}$; RQ-MIN and RQ-MAX constitute the acceptable error for a 95% confidence limit according to Student's *t*-test]. When either an RQ-MIN or RQ-MAX level was 25% less or more, respectively, of the corresponding $2^{-\Delta\Delta Ct}$ value, we analyzed the sample until the technical variation achieved satisfactory within the range. mRNA levels are shown with relative values based on those from a randomly selected patient sample in this cohort.